Viral and Rickettsial Diseases

Laboratory Methods in Diagnosis

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• *Factors contributing to the development of viral diagnostic services have been: (1) technical advances, and (2) increasing demand for services due to relative and actual increases in the prevalence of these illnesses. This increase has been both relative, as in the case of diphtheria, etc., and actual as in the cases of poliomyelitis. Technical advances have been numerous and frequent. One of the most spectacular has been the development of methods for the culture of living cells for the propagation of viruses. Emphasis must be placed on the fact that the diagnosis of many viral diseases requires close teamwork between local, state, federal, and privately supported agencies. Laboratory procedures remain expensive but are frequently the only way to determine the exact nature of a particular illness. The available or practical procedures are emphasized in this discussion.*

THE DEVELOPMENT of laboratory procedures for the diagnosis of viral and rickettsial diseases has followed the general pattern established for other infectious diseases. At the beginning of this century the main laboratory diagnostic services available were the detection of rabies in dogs, the complement-fixation test for syphilis and the cultural methods for the diagnosis of diphtheria and tuberculosis. This state of affairs prevailed until after World War I. The large state-supported "institutes" of Europe were engaged primarily in the manufacture of vaccines and therapeutic sera and their excursions into laboratory diagnostic "service" were made chiefly to investigate veterinary or human diseases of economic importance or of other national concern. No "service" as we think of it today was available.

Many of the immediately practical procedures were performed by practicing physicians, but with the increasing complexity of these procedures that became impossible. Procedures which were most useful and not prohibitively expensive for the individual patient were performed in private hospital laboratories and in private clinical laboratories. There remained, however, a large group of tests so expensive that they could be made generally available only through federal and state agencies, and those tests as well as the more usual procedures were assumed gradually by city, county, state and federal laboratories in this country.

With the exception of rabies detection, viral laboratory procedures were limited to the recovery and identification of an infectious agent in suitable experimental animals. About 25 years ago the serum neutralization test (which had been proposed and first used experimentally by Landsteiner about 15 years earlier) was developed for yellow fever. Subsequently this technique proved applicable to a variety of viral diseases. However, the requirement of 50 or so mice to carry out a test of this type for one virus and with but a single pair of blood specimens made development of *in vitro* techniques extremely desirable. Thus the application of complement-fixation was broadened with the development of satisfactory viral and rickettsial complement-fixation antigens.

In California, viral diagnostic services were first provided in connection with investigative programs of various university and private laboratories; the Hooper Foundation of the University of California was particularly active in this field. In the late 1930's a laboratory facility was established in Berkeley for the investigation of influenza and related epidemic respiratory diseases. This laboratory, which initially was privately supported for field research purposes, developed into a state-wide, publicly supported diagnostic service.

The factors contributing to the development of viral diagnostic services have been: Technical advances, acceptance of responsibility for provision of such service by public agencies, and increasing demand for services due to increased importance of viral diseases. This increase has been both relative, because of declining prevalence of such diseases as diphtheria, tuberculosis and bacterial pneumonia, and actual, as in the case of poliomyelitis.

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Local service needs should be met in so far as possible, by local laboratory facilities, but local service must of necessity be limited in scope because of the burden of overhead necessary for the maintenance of comprehensive service and research facilities essential for a complete viral and rickettsial diagnostic program.

METHODS

The methods developed for the specific diagnosis of other infectious diseases are almost all adaptable in one way or another to the diagnosis of viral and rickettsial infections. However, three limiting factors—the small mass of viruses and of rickettsiae, the lack of detectable morphological differences, and the requirement for a living host cell for growth and manifestation of pathogenicity—have imposed formidable practical limitations. In the conventional agglutination test, the Widal for example, the typhoid bacilli comprise the great proportion of the agglutinated mass, the visible endpoint, antibody contributing only a small amount; and, in the precipitin and flocculation tests, the antigens although of small size are readily available in large quantities of concentrated material. On the other hand, although specific aggregation or agglutination of the rickettsiae and larger viruses by antibody is feasible and is actually employed on occasion in investigative studies, the preparation of concentrated suspensions of the infectious agents is so difficult as to preclude practical diagnostic use. The necessity for living systems has been partially overcome, first through the use of small, relatively inexpensive animals (mice, and more recently baby mice), second, through the use of embryonated eggs, and third and most recently through improved methods of tissue culture. The available techniques and the applications are listed in Table 1.

The complement-fixation technique is an old and familiar procedure and its employment in viral and rickettsial diagnosis is not materially different from its use in syphilis. Unlike the syphilis tests, however, the viral and rickettsial antigens are *specific*. There are some instances of a general cross-reactivity, such as between Rocky Mountain spotted fever and rickettsialpox, but these are not troublesome. Rather, these cross-reactions can be useful, as for example in the use of the soluble typhus antigen for screening purposes rather than the more expensive but specific washed rickettsial antigens, to distinguish classical and murine typhus antibodies. Modern development of dried complement,

TABLE 1.—*Summary of diagnostic procedures for viral and rickettsial diseases*

Type of Examination	CLASS OF DISEASE				
	Respiratory	Central Nervous System	Cutaneous Systemic	Eye	Other
Complement-fixation	Q fever Psittacosis Influenza	Encephalitis Mumps LCM Herpes simplex Colorado tick fever	Typhus Spotted fever Herpes simplex Rickettsialpox	Herpes simplex	LGV Mumps
Hemagglutination inhibition	Influenza	Mumps		Newcastle disease	
Neutralization	Influenza*	Encephalitis LCM Poliomyelitis	Variola vaccinia Herpes		Yellow fever
Nonspecific agglutination	Primary atypical pneumonia		Typhus Spotted fever Scrub typhus		Infectious mononucleosis
Direct microscopic		Rabies	Variola	Trachoma Inclusion blenorrhea	
Cultural	Influenza Epidemic pleurodynia and herpangina	Encephalitis Mumps LCM Colorado tick fever Herpes simplex Rabies Poliomyelitis Coxsackie B	Variola Typhus Spotted fever Rickettsialpox Scrub typhus		Mumps
No satisfactory test	ARD (common cold)		Varicella Herpes zoster Measles Rubella		Viral enteritis Hepatitis

*Available but not generally used because of practical limitations.

Key to Abbreviations: ARD=Acute respiratory disease; LGV=Lymphogranuloma venereal; LCM=Lymphocytic choriomeningitis.

uniform and high titer sheep-cell hemolysin, and noninfectious, reliable antigens has made viral and rickettsial complement-fixation techniques adaptable to local laboratory use.

Fortuitously, the complement-fixation is proving especially useful in those diseases caused by a number of closely related agents. For example, the large number of antigenically different strains of influenza A and A′ viruses require multiple antigens for neutralization and hemagglutination inhibition tests, but a common complement-fixation antigen reacts with antibodies produced by any of the strains. Furthermore, high levels of complement-fixation antibody appear to be closely associated with the active phase of infection with a particular agent, do not persist long after recovery, and are not ordinarily seen in high titer following vaccination.

Hemagglutination inhibition, based on the property of specific immune serum to prevent clumping of erythrocytes by various viruses, has become a very popular procedure particularly in the epidemiologic investigation of influenza. This technique has also been found useful in the diagnosis of mumps and certain diseases of veterinary importance. The technique is simple and reproducible and can be carried out with a minimum of equipment. The readiness with which this test detects minor antigenic variation limits its utility and its scope (Table 1). Hemagglutination inhibition techniques have been proposed for equine and St. Louis encephalitis, but at present they are of such technical complexity that they are not practical.

Neutralization of viruses by the corresponding specific antibodies has the prestige of seniority over the other techniques. The use of animals has been traditional as a device to detect residual virus in mixtures of virus and serum, but animal inoculation has become more general with the adoption of chick embryo, infantile animals and tissue cultures as testing tools. Development of the tissue culture technique has been meteoric after a long lag period, and today practical tissue culture procedures are almost within the scope of the routine testing laboratory.

The nonspecific tests, best exemplified by the Weil-Felix proteus agglutination in the diagnosis of typhus fever, have had long usefulness. But wherever the specific antigen is feasible and reasonable in cost, its use is recommended over the nonspecific antigen. Academically this is highly desirable but practically is not always the best procedure. For example, in early-treated cases of typhus and Rocky Mountain spotted fever the complement-fixation reactivity of the early convalescent phase serum may be inconclusively low or absent, whereas the proteus OX19 or OX2 agglutination to high titer by the same serum may permit a diagnosis. Infectious mononucleosis is included in the table on the assumption that it is caused by a virus, although this has not been proved. The sheep-cell agglutination in this instance is analogous, perhaps, to the Weil-Felix test before the etiologic agent of typhus fever had been defined. Agglutination of human erythrocytes at cold temperatures by early convalescent phase serum has been extremely useful in the definition of certain primary atypical pneumonias. This phenomenon is an example of a nonspecific autoantibody reaction.

Direct microscopic methods generally have as their purpose the demonstration of characteristic inclusion bodies and viral particles in cells or exudate obtained directly from the lesions of a disease. Diseases that lend themselves to direct microscopic methods are rabies, variola, herpes simplex, trachoma and inclusion blenorrhea. Varicella-herpes zoster infections are not practically diagnosed thus, although they may enter the differential diagnostic possibilities of certain of the other diseases mentioned and may be diagnosed by exclusion. Inclusion body detection has been suggested as a diagnostic procedure for examination of sputum and material washed from the throat in certain respiratory illnesses, but so far has been of no practical value. A drawback to this method is obvious in rabies diagnosis in dogs—namely that inclusions resembling the Negri body may be observed in animals dead of other diseases, and that absence of inclusions in the material examined does not absolutely deny the diagnosis of rabies infection.

Cultural procedures, as has been mentioned, entail the transmission of the infectious agent to a susceptible host system: An intact animal, avian embryo or tissue culture. In those instances in which the changes induced in the experimental system are specific—rabies, for example—immediate identification of the agent may be possible as soon as disease appears. However, in most viral infections, whether of animals or cells in culture, the changes are nonspecific and it is necessary to identify the specific agent by neutralization, complement-fixation or protection tests with known specific antisera. Even in rabies this procedure is occasionally necessary. In spite of these limitations, culture of the infectious agent is very useful in the laboratory diagnosis of poliomyelitis (tissue culture), the Coxsackie infections (infant mice), smallpox (chick embryo) and herpes simplex (chick embryo and infant mice), as well as a sound approach to the ultimate discovery of practical methods for the diagnosis of other diseases.

HANDLING THE SPECIMENS

Identification. This simple and obvious necessity is overlooked frequently; a satisfactory specimen may arrive in good condition at the laboratory—but without the patient's name or the physician's name or either. A brief summary of the clinical history, including the date of onset and date of bleeding or other sampling is likewise of great assistance in interpreting laboratory test results.

Blood Specimens for Serologic Tests. Glassware for blood specimens should be clean, dry, and preferably sterile. Care should be exercised to remove all of the cleaning agent because most of them interfere with serologic tests and are also toxic to viruses, to other microorganisms, to laboratory animals and to tissue cultures. For example, chromate ion (in the commonly used chromic acid cleaning solution) is extremely adherent to glass and will poison viruses stored in such glass. Sodium lauryl sulfate, on the other hand, can be rinsed out of glassware readily and therefore is better for cleaning tissue culture glass. Sterility of the specimen, always desirable, is essential for neutralization studies and for most animal work. Blood bottles or tubes should be tightly stoppered, preferably with rubber stoppers. In the case of air-mailed tubes it is wise to tape the stoppers securely to prevent their popping out at low air pressure. Postal regulations call for a double container, an inner one of metal, an outer one of fiber or cardboard, to prevent damage to other mail and hazard to postal employees. Blood specimens for serologic tests should be kept cool or refrigerated, but not frozen. Frozen and thawed blood cannot be tested by most techniques; frozen and thawed serum, however, can be examined serologically.

Biopsy and Necropsy Specimens. Blood, tissue for biopsy, cerebrospinal fluid, throat washings, sputum, vesicle fluid or crusts should without exception be preserved by rapid freezing, and shipped in dry ice. Some viruses are relatively stable in water or in 50 per cent glycerine (for example, poliomyelitis, Coxsackie, herpes), but many are extremely labile. Smallpox virus is uniquely resistant to drying; therefore dried crusts or pox fluid may be shipped long distances or stored for relatively long periods over drying agents without loss of virus. On the other hand, poliomyelitis virus, which is relatively stable in water, is rapidly destroyed by drying. When dry ice is not available it is best to ship in wet ice according to the procedure used to transport dog heads. It is poor practice to store such materials in the home or office freezer or freezing compartment because of the alternate slow freezing and slow thawing which occurs during such storage.

Surgical aseptic technique should be the guiding principle in obtaining specimens at necropsy for purposes of viral or rickettsial isolation. This is likewise a prudent precaution for protection of personnel and the environment against possible dangerous and insidious contamination. Such specimens should be preserved and transported with precautions outlined above.

Laboratories commissioned to provide viral diagnostic services for physicians in California are:

1. The Virus Laboratory, California State Department of Public Health, Berkeley, California (Statewide service).
2. The Virus Diagnostic Laboratory, United States Public Health Service, Montgomery, Alabama (Specimens should be submitted through the State Virus Laboratory).
3. The Virus Laboratory, Los Angeles City Health Department, Los Angeles (for physicians in the city of Los Angeles including Los Angeles County Hospital).
4. For Military and V. A. installations, the Sixth Army Area Laboratory, Fort Baker, and the Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School, Washington 12, D. C.
5. An increasing number of city and county laboratories are providing serological tests as commercial antigens become available. In areas other than those mentioned, the physician is advised to consult his local health officer.

Other qualified laboratories which might be interested in unusual infections from an investigative point of view are:

1. The University of California Medical Center, San Francisco
   (a) The Hooper Foundation (particularly psittacosis and ornithosis).
   (b) The Proctor Foundation (ophthalmic infections).
   (c) The Department of Pediatrics (smallpox).
2. The University of California Medical Center, Los Angeles
   (a) The Division of Virology, Department of Infectious Diseases and the Central Hospital Laboratories.
   (b) The Department of Pediatrics (Respiratory Infections in the newborn).
3. The College of Medical Evangelists
   Department of Pediatrics
   Los Angeles County Hospital.

DISCUSSION

Of the procedures listed, the *in vitro* serological procedures are by far the most economical; the complement-fixation test is particularly useful and is the most widely used test for those diseases for which satisfactory complement-fixation antigens are available and in which complement-fixation antibodies appear. A particular advantage is that whereas neutralizing and hemagglutination inhibiting antibodies may persist for months and years following immunization or infection, complement-fixation antibodies tend to fall to appreciably lower levels in weeks or months following most diseases.

Practically, serial specimens, one collected as early in the course of the infection as possible and one or more collected during convalescence, are always desirable. However, high levels of complement-fixation antibody can often be regarded as presumptive evidence of recent infection even though an increase cannot be demonstrated. The problem of the early or "base line" serum is a very real one because obviously the physician may not suspect a viral infection until late in the course of the disease.

The best results in virus diagnostic work are obtained when there is close collaboration between the physician and the laboratory. One of the most frustrating experiences in virus diagnostic work is to receive a specimen with a "blanket" request for "virus diagnostic studies"; obviously, even in tax-supported institutions one cannot set up a battery of tests like "febrile agglutinins" for all of the viral infections when the tests for a single agent may cost fifty to a hundred dollars. If the physician cannot make a specific request himself, or cannot telephone the laboratory, the next best alternative is to send a brief clinical abstract of the case so that the director of the laboratory may select the appropriate tests.

Emphasis herein on the limitations of virus diagnostic tests will not, it is to be hoped, be completely discouraging. The situation is infinitely more optimistic now than it was 15 years ago and we can look forward to the availability of more and more useful procedures.

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